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TOPICAL REVIEW

Biological applications of colloidal nanocrystals

Wolfgang J Parak[1,2,4], Daniele Gerion[1,2], Teresa Pellegrino[1], Daniela Zanchet[1], Christine Micheel[1], Shara C Williams[1], Rosanne Boudreau[2], Mark A Le Gros[2], Carolyn A Larabell[2,3] and A Paul Alivisatos[1,2]

[1] Department of Chemistry, University of California Berkeley, Berkeley, CA 94720, USA
[2] Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
[3] Department of Anatomy, University of California San Francisco, San Francisco, CA, USA

E-mail: Wolfgang.Parak@physik.uni-muenchen.de

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Abstract
Due to their interesting properties, research on colloidal nanocrystals has moved in the last few years from fundamental research to first applications in materials science and life sciences. In this review some recent biological applications of colloidal nanocrystals are discussed, without going into biological or chemical details. First, the properties of colloidal nanocrystals and how they can be synthesized are described. Second, the conjugation of nanocrystals with biological molecules is discussed. And third, three different biological applications are introduced: (i) the arrangement of nanocrystal–oligonucleotide conjugates using molecular scaffolds such as single-stranded DNA, (ii) the use of nanocrystal–protein conjugates as fluorescent probes for cellular imaging, and (iii) a motility assay based on the uptake of nanocrystals by living cells.

1. Introduction

Certainly, one important branch of nanotechnology is nanobiotechnology. What does an interaction between nanotechnology, e.g. the design of artificial nanometre structures, and biological or medical science look like? There are two directions in which this interaction can be considered. First, structures created with nanotechnology can be used as highly sophisticated scopes, machines or materials in biology and/or medicine. Second, biological molecules can be used to assemble nanoscale structures.

One dream of nanotechnology certainly is to develop tools and machines to investigate and manipulate biological structures on a nanometre scale. These machines do not necessarily refer to Drexler's nano-robots [1, 2], which are supposed to circulate around in our veins repairing defects. Nanotechnology is more than scaling down macroscopic machines to nanometre size. This is especially due to the fact that many effects on the nanometre scale can no longer be described with the laws of classical mechanics but instead follow the rules of quantum mechanics. Therefore a machine for drilling holes, for example, will look different on the nanometre scale compared to macroscopic drills. Some other machines we know from the macroscopic world will not even have an equivalent on the nanometre scale. Smalley [3] pointed out the impossibility of nano-fingers that could assemble large molecules by adding atom by atom. On the other hand we can expect many novel effects on the nanoscale which will allow for creating machines and materials with unique properties without macroscopic counterparts. How can nanotechnology help to solve biological and/or medical problems? One realistic application is, for example, the use of nanostructured materials as artificial bones [4]. Furthermore, colloidal semiconductor nanocrystals are used as fluorescent probes to label cells [5–14] and chemical libraries [15–17].

[4] Address for correspondence: Centre for Nanoscience, Ludwig Maximilians University, Amalienstraße 54, 80799 München, Germany.

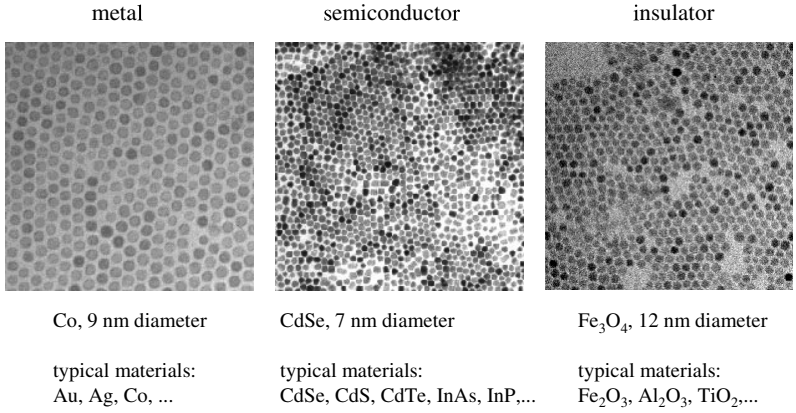

Figure 1. Colloidal nanocrystals of different materials. Colloidal nanocrystals can be synthesized from metallic, semiconductor or insulating materials. Low-resolution TEM images from nanocrystals made out of Co, CdSe and Fe_3O_4 arranged on a TEM grid are shown. The images were recorded by V Puntes, L Manna and M Casula. Each sphere corresponds to one single nanocrystal.

Another example is an atomic force microscope (AFM) based sensor, which detects biological molecules by binding them to a tiny cantilever, therefore tuning its resonance frequency [18–20]. As a final example, the miniaturization of a wide variety of laboratory apparatus to the size of a silicon chip can be mentioned. In this way the speed and throughput of classical biochemical methods, such as gel electrophoresis and polymerase chain reaction (PCR), can be increased [21]. Although it is obvious from these examples that nano-machines look different from their macroscopic equivalents they prove that nanotechnology has already contributed important tools to investigate and manipulate biological nano-objects.

Regarding the interaction from the other direction, biological molecules can be harnessed for the creation of nanostructures and can be used as a sort of 'glue' to assemble nanoscale building blocks [22–24]. This idea starts with the generation of artificial nanostructures, in which molecules are arranged in a self-assembly process [25–29] enabling the formation of complex patterns [9, 30]. The basis of processes like this is the principle of molecular recognition. Many biological molecules, such as DNA, can bind to other molecules in a lock-and-key manner with very high selectivity and specificity. As an example, the sequences of single-stranded oligonucleotides can be chosen such that binding to other, partly complementary single-stranded oligonucleotides causes the formation of complex patterns such as two-dimensional crystals or cubes [31–35]. Watson–Crick base pairing stabilizes these patterns. Such DNA patterns can be regarded as nanoscale scaffolds, which act as templates for the organized addition of other structures [36, 37]. The general idea is to use the molecular recognition capability of biomolecules to facilitate the arrangement of nanoscale building blocks in a parallel process, rather than having to assemble these blocks sequentially or individually. It is, for example, only of limited use to be able to use nanocrystals as tiny transistors, without having in addition a technology that enables their arrangement and connection. Besides using biological molecules as nano-templates for the generation of complex structures, biological molecules can also be used as functional elements. One example is the transport of electric charge in nanowires of metallized DNA [38–40].

In this review the use of colloidal nanocrystals as nanometre scale building blocks will be discussed. In order to use the nanocrystals for biological or medical applications, they first need to be conjugated to biological molecules. In this way, the nanocrystals can be considered building blocks, whereas the biological molecules fulfil the role of a multifunctional glue to arrange and connect the building blocks. After a general description of the synthesis of nanocrystals and their bioconjugation, three types of applications will be described. First, the programmable DNA-directed arrangement of nanocrystals, then the use of semiconductor nanocrystals as fluorescent probes for the labelling of cells, and finally the interaction of nanocrystals with living cells will be discussed.

2. Nanocrystals: what they are and how they can be made

2.1. What are nanocrystals?

Nanocrystals are crystalline clusters of a few hundred to a few thousand atoms with sizes of a few nanometres. Although more complex than individual atoms, their properties are different from bulk crystals. Due to their small size, much of their chemical and physical properties are dominated by their surfaces and not by their bulk volume [41–43]. Nanocrystals can be synthesized from metallic materials such as gold [44–47], silver [48, 49] or cobalt [50–53], from semiconductor materials such as cadmium sulfide [54] cadmium selenide [54–61], cadmium telluride [54, 61–64], gallium arsenide [65] or indium phosphide [66, 67], and from insulators such as iron oxide [68–70] or titanium oxide [71] (see figure 1). Three properties are important for the quality of colloidal nanocrystals. First, nanocrystals obviously should be crystalline and thus preferentially consist of only one domain. Second, their size distribution should be as narrow as possible and third, there should be a unique, uniform shape to nanocrystals in a particular sample. Colloidal nanocrystals are dispersed in a solvent and should be stabilized in a way that prevents agglomeration. Besides spherical nanocrystals, more complex geometries such as rods or cylinders [51, 52, 72–76], prisms [77] and tetrapods [78] can be synthesized in a controlled way.

2.2. Some quantum-physical properties

Because of their small size of only a few nanometres, which is of the same order of magnitude as the de Broglie wavelength of electrons and holes at room temperature, the states of free charge carriers in nanocrystals are quantized. For spherical nanocrystals, in which free electrons and holes are confined in all three directions, the movement of charge carriers is completely determined by quantum mechanics. The nanocrystals, therefore, are also often called quantum dots. Analogous to a particle-in-a-box model, this results in discrete energy levels, the spacing of which depend on the size of the nanocrystal [79–85]. Because of the similarity between the discrete energy levels of quantum dots and the discrete energy levels of atoms, quantum dots are often thought of as artificial atoms [86, 87]. Since the energy levels are determined by the size of the nanocrystal, they can be tuned in a controlled way by synthesizing nanocrystals of different diameters [42, 82]. The smaller a nanocrystal, the larger the spacing between its energy levels will be. For semiconductor nanocrystals, this involves a size-dependent band gap. Analogous to atoms, charge carriers are excited to upper energy levels if nanocrystals are excited optically. If, as is the case for many semiconductors, the band gap of the bulk semiconductor is in the infrared, the wavelength of the fluorescence light emitted when excited charge carriers fall back to the ground state will be in the visible spectral range. The smaller the nanocrystal is, the larger the spacing between the energy levels and therefore the larger the energy gap and, thus, the shorter the wavelength of the fluorescence. Small (e.g. 2.5 nm diameter) CdSe nanocrystals have green fluorescence; large ones (e.g. 7 nm diameter) are fluorescent in the red (see figure 2) [5]. By controlled adjustment of the size during the synthesis of semiconductor nanocrystals basically all fluorescence colours in the visible region can be obtained [82]. Compared to organic fluorescent dyes, nanocrystals offer some advantages. First, they suffer less from photobleaching. Second, they can be excited at any wavelength that is shorter than the wavelength of their fluorescence; also, there is no red-tail in the emission spectrum [5, 88]. Non-spherical, for example rod-shaped nanocrystals, can also fluoresce. Because of their asymmetric shape, the emitted light is polarized, which offers new possibilities [75, 89]. In the following section two types of nanocrystals will be discussed in particular: colloidal Au and CdSe/ZnS.

2.3. Synthesis of colloidal nanocrystals (Au, CdSe/ZnS)

The most prominent material for colloidal metal nanocrystals is certainly gold. Colloidal gold can be synthesized with high quality in organic [46, 90, 91] as well as in aqueous solution [44, 92].

One of the most frequently used materials for the synthesis of colloidal semiconductor nanocrystals is cadmium selenide. In order to obtain a good size distribution, the synthesis is generally performed in organic solvent at high temperature. A typical experimental set-up is shown in figure 2. Trioctylphosphine oxide (TOPO) is heated and the liquid precursors (dimethyl cadmium and selenium powder dissolved in tributylphosphine), similar to those used in chemical vapour deposition (CVD), are quickly injected with a syringe [59].

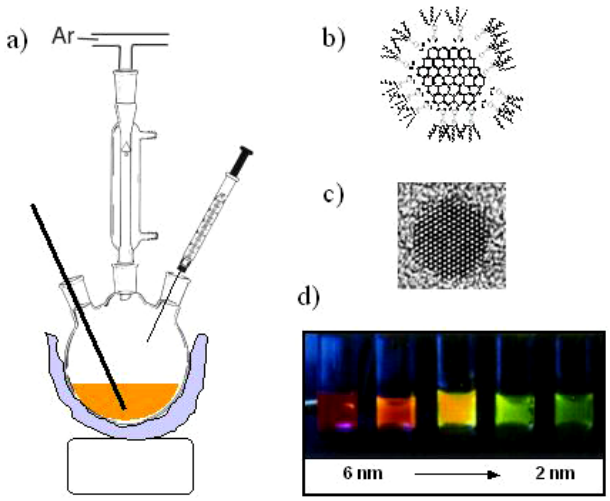

Figure 2. Synthesis of colloidal nanocrystals. (a) Typical set-up. The surfactant in a three-neck flask is heated in an Ar atmosphere. The temperature is measured with a thermometer in the solution and the solution is mixed with a stirrer that is positioned below the heating mantle around the flask. The precursors are quickly injected with a syringe. (b) Cartoon of a nanocrystal. The surface of each particle composed of a CdSe lattice is covered with a layer of surfactant molecules, such as TOPO. (c) High resolution TEM image of one CdSe nanocrystal. Each dot corresponds to a column of atoms in the CdSe lattice. The TOPO layer is not visible due to its low contrast. The diameter of the nanocrystal is about 5 nm. (d) Colloidal CdSe nanocrystals dissolved in toluene. Each vial contains CdSe nanocrystals of a different size, ranging from about 2 to 6 nm. All solutions were excited with a hand-held UV lamp and a photograph of the fluorescence was recorded. The small (2 nm) nanocrystals emit green, and the large (6 nm) ones emit red light.

CdSe nanocrystals immediately start to nucleate and the initially colourless solvent becomes coloured. The desired size of the nanocrystals can be adjusted by changing the amount of injected precursors and the time they are grown in the hot TOPO [59]. The nanocrystals obtained in this way are hydrophobic, since they are covered with a surfactant layer (see figure 2). This layer of TOPO molecules stabilizes the nanocrystals in solvents like toluene or chloroform and prevents agglomeration. According to the size of the synthesized nanocrystals, fluorescence in practically all colours of the visible spectrum can be obtained. Frequently, to increase the quantum yield, i.e. to reduce non-radiative processes at the nanocrystal surface, a shell of a semiconductor material with a higher band gap such as ZnS is epitaxially grown around the CdSe cores. Such CdSe/ZnS core/shell nanocrystals reach quantum yields of 70–80% [93–97].

2.4. Water-soluble colloidal nanocrystals

Since biological processes are typically situated in an aqueous environment, a hydrophilic nanocrystal surface is desired for reactions with biological molecules. Colloidal gold is commercially available in aqueous solution with a good size distribution (Sigma-Aldrich, Ted Pella). However, these particles are not stable in micromolar concentrations in electrolytic solutions. As soon as a significant amount of NaCl is introduced, these particles aggregate and the colour of the solution changes from red to black. In

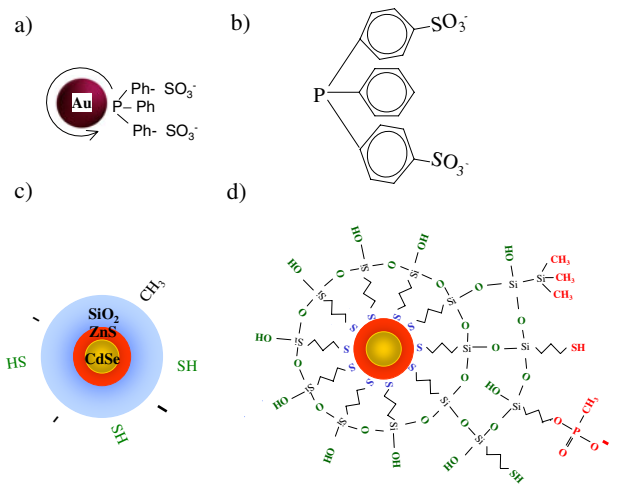

Figure 3. Water-soluble nanocrystals. (a) Nanocrystalline gold particles can be stabilized in aqueous solution by coating them with a layer of phosphine molecules. The phosphine is adsorbed on the gold surface through its phosphorus atom. Phosphine coated Au nanocrystals are negatively charged and therefore repel each other. (b) Structure of a typical phosphine molecule used for particle stabilization (bis(p-sulfonatophenyl)phenylphosphine). (c) Silanized CdSe/ZnS nanocrystal. The CdSe/ZnS core is embedded in a glass-like shell. The surface of the shell exposes thiol (–SH) groups, negatively charged phosphonate groups (−), and methyl ($–CH_3$) groups that originate from the chlorotrimethylsilane, which is used to stop the cross-linking process. Alternatively, nanocrystals with positively charged ammonium groups (+) or neutral polyethylene groups can be synthesized (not shown). (d) Details of the silanized CdSe/ZnS particle shown in (c). The first layer of silane molecules is adsorbed onto the ZnS surface via thiol (–SH) groups. The silane layers are cross-linked via siloxane (–Si–O–Si–) bonds. The outside exposes thiols, phosphonates and methyl groups.

order to stabilize the particles, they are coated with surfactant molecules. If molecules such as phosphines (e.g. bis(p-sulfonatophenyl)phenylphosphine) are added to a solution of colloidal gold, they adsorb onto the nanocrystal surface (see figure 3). Since phosphine molecules are negatively charged, the nanocrystals now strongly repel each other electrostatically; this Coulombic interaction is stronger than the van der Waals attraction between particles. Phosphine stabilized Au nanocrystals are stable even in solutions containing moderate amounts of salts (e.g. 50 mM NaCl) [45, 98, 99]. Besides phosphines, other surfactant molecules that introduce negative charge, for example carboxyls, are also used for stabilization [100, 101].

Semiconductor nanocrystals composed of a variety of materials can be directly grown in aqueous solutions, such as CdS [63, 102–109] and CdTe [63, 110, 111]. However, the size distribution and quantum yield of these particles in general is worse than that of particles grown in organic surfactants like TOPO. This review therefore focuses on CdSe/ZnS core/shell nanocrystals grown in TOPO as described above. Their surface is hydrophobic and therefore they are not directly soluble in aqueous solution. The easiest way to obtain a hydrophilic surface is by exchanging the hydrophobic TOPO surfactant molecules with bifunctional molecules that are hydrophilic on one end and bind to ZnS with the other end. Commonly, thiols (–SH) are used as ZnS-binding groups, and carboxyl (–COOH) groups are a prominent example for hydrophilic groups. In this way, TOPO-capped CdSe/ZnS nanocrystals can be transferred into aqueous solution by exchanging the TOPO adsorbed on their surface for a layer of a mercaptocarbonic acid (HS–· · ·–COOH), such as mercaptoacetic acid, mercaptopropionic acid or mercaptodecanoic acid [6, 88, 112–117]. Carboxyl groups are negatively charged at neutral pH. Nanocrystals capped with carboxyl groups therefore repel each other electrostatically. In electrolytic solution the Coulombic interaction is shielded and the particles precipitate at salt concentrations of a few hundred millimolar [88]. Unfortunately, thiol–ZnS bonds are far less stable than thiol–Au bonds. The water solubility of CdSe/ZnS nanocrystals capped with mercaptocarbonic acids is therefore limited [117]. Thiol–ZnS bonds are dynamic, which means that the thiol ligands bind and unbind in a dynamic equilibrium. However, as soon as these nanocrystals are dialyzed against pure buffer or water over a longer period, they start to precipitate, since all free thiol ligands have disappeared. Enhanced stability can be achieved by using mercaptocarbonic acids with two instead of just one thiol group [7, 118, 119].

A more laborious but more stable method of converting CdSe/ZnS nanocrystals into water-soluble particles is surface silanization. Surface silanization is basically growing a glass shell around the particles (see figure 3) [5, 88, 120–129]. The first step in the surface silanization of CdSe/ZnS nanocrystals is to bind a priming layer of mercapto-trimethoxysilane molecules to the surface of the nanocrystals via thiol/ZnS bonds. The trimethoxysilane groups can be cross-linked, which greatly stabilizes the silane layer on the CdSe/ZnS surface. In the second step, hydrophilic trimethoxysilane molecules are added. By cross-linking the trimethoxysilane groups through the formation of siloxane bonds, this shell is connected with the priming layer [5, 88, 128]. Two types of hydrophilic trimethoxysilanes have been used to stabilize nanoparticles in aqueous solution. One of these types is the use of silanes that are negatively or positively charged at neutral pH in aqueous solution, such as silanes with phosphonate or ammonium head groups [128]. In this way the particles repel each other electrostatically and they are soluble at micromolar concentrations in buffer solutions of a few hundred millimolar ionic strength. The other possibility is to use silane molecules with long hydrophilic chains, such as polyethylene glycol (PEG) [128, 130]. Since PEG-silane coated particles repel each other by steric interaction, they have almost no electrical charge. By varying the composition of the hydrophilic trimethoxysilanes, water-soluble nanocrystals with every desired charge, ranging from positive to negative, can be obtained [128]. In the second step of the silanization, mercapto-trimethoxysilanes are added again. They are incorporated into the silica shell by formation of siloxane bonds and some of the mercapto groups point towards the aqueous solution in which the nanocrystals are dissolved. These thiol groups act as anchors for further reactions in which biomolecules are covalently coupled to the surface of the nanocrystals. In summary, the main advantage of surface silanization, compared to other procedures for stabilizing nanoparticles in aqueous solutions, is that the ligand layer providing water solubility is highly cross-linked and therefore extremely stable.

Another possibility for stabilization of CdSe/ZnS nanocrystals in aqueous solutions might be to cover them

with a polymer layer that is adsorbed to the particle surface. This strategy has already been successfully applied for ZnO nanoparticles [131].

3. Bioconjugation of nanocrystals

3.1. Attaching oligonucleotides to colloidal Au nanocrystals

Although oligonucleotides can be attached to the surface of colloidal gold nanocrystals by simple adsorption [132] or via a biotin–avidin linkage, where the avidin is adsorbed on the particle surface [133], the most commonly used method is a linkage via thiol–gold bonds [37, 134–146]. Thiol-modified oligonucleotides stick with high affinity to gold surfaces. In this way, when gold nanocrystals are incubated in a solution of thiol-modified oligonucleotides, the oligonucleotides will bind to the surface of the nanocrystal (see figure 4). The attachment via thiol linkage to nanocrystals is much stronger and more efficient than nonspecific adsorption [143]. Unfortunately, the number of oligonucleotides attached per nanoparticle cannot be directly controlled. If, for example, oligonucleotides are added to gold nanocrystals in a stoichiometric ratio of 3:1 and a binding efficiency of 100% is assumed, the solution would always contain a distribution of gold–oligonucleotides: besides gold nanoparticles with 3 oligonucleotides there would also be some gold nanoparticles with 2 or 4 oligonucleotides. However, gold nanocrystals with a controlled number of oligonucleotides attached can be isolated using gel electrophoresis (see figure 4) [143]. An agarose gel is basically a porous polymer matrix, where the average pore size decreases with increasing agarose percentage [147, 148]. Smaller particles experience less resistance from the gel and can move more rapidly. During gel electrophoresis a voltage is applied along the gel. Both oligonucleotides and phosphine-coated gold nanocrystals are negatively charged. If gold–oligonucleotide conjugate samples are loaded in the gel, they will migrate towards the positive electrode. Since conjugates with, for example, two oligonucleotides attached per gold particle are larger than conjugates with just one oligonucleotide attached per particle, they migrate less. In this way, conjugates with a different number of oligonucleotides bound per Au nanocrystal can be separated by gel electrophoresis (see figure 4) [143, 149]. The conjugates can be isolated from the gel and can be used for experiments that require a controlled number of oligonucleotides per particle.

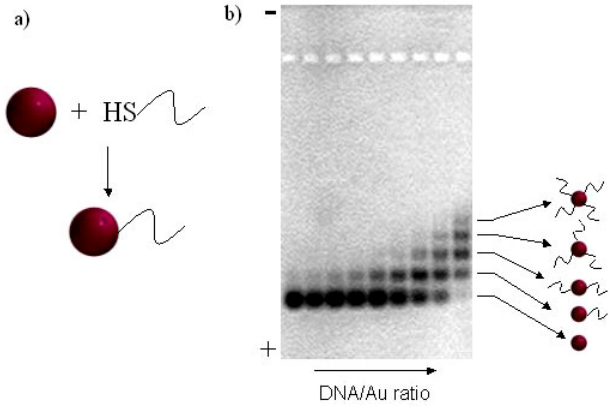

Figure 4. Conjugation of gold nanocrystals with oligonucleotides. (a) Oligonucleotides modified with thiol (–SH) groups directly bind to the surface of gold nanocrystals. (b) Separation of gold–oligonucleotide conjugates with gel electrophoresis. 100 base oligonucleotides are added in different stoichiometric amounts to 10 nm diameter Au nanocrystals and are incubated for 2 h. The samples are loaded in the wells of a 2% agarose gel (white squares that can be seen at the top of the image, close to the '−' electrode). A voltage (100 V $\Leftrightarrow$ 6.7 V cm^{-1}) is applied along the gel for 1 h. The conjugates migrate towards the '+' electrode. The higher the oligonucleotide (DNA) to Au nanocrystal ratio, the more slowly the conjugates migrate. Discrete bands can be resolved. The first, second, third, etc, band can be ascribed to Au nanocrystals decorated with zero, one, two, etc, oligonucleotides per Au nanocrystal.
(This figure is in colour only in the electronic version)

3.2. Attaching oligonucleotides and other biological molecules to colloidal silanized CdSe/ZnS nanocrystals

Several strategies for the conjugation of water-soluble semiconductor nanocrystals with biological molecules have been reported. They include adsorption [104, 150], linkage via mercapto groups [112, 114, 116], electrostatic interaction [118, 119] and covalent linkage [5–7, 108, 111, 115, 128, 151]. For reasons of stability, covalent linkage seems to be the most desirable method. Oligonucleotides can be covalently attached to silanized nanocrystals with bifunctional crosslinker molecules [128]. Bifunctional crosslinker molecules are molecules with two reactive ends, which can be used to connect two particles or molecules. Many different crosslinker molecules are commercially available, which are reactive toward groups such as –COOH (carboxyl), –NH_2 (amine), or –SH (thiol, mercapto). Silanized nanocrystals as described above expose mercapto (–SH) groups on their surface that can act as anchors for further reactions. Oligonucleotides can be modified with different functional groups, such as amines (–NH_2). In this way, amine-modified (–NH_2) oligonucleotides can be bound to thiol-bearing (–SH) silanized nanocrystals with a bifunctional crosslinker that is reactive on one end towards amine groups and on the other end towards thiol groups (see figure 5). An essential step in reactions like this is purification of the nanocrystals from excess crosslinker molecules [128]. As in the case of gold–oligonucleotide conjugates, the attachment of oligonucleotides to silanized CdSe/ZnS nanocrystals can be probed with gel electrophoresis (see figure 5). CdSe/ZnS nanocrystals stabilized with PEG groups are almost neutral [128]. Therefore, they barely migrate in the electric field applied along an agarose gel. If negatively charged oligonucleotides are bound, the nanocrystal–oligonucleotide conjugate becomes negatively charged and migrates towards the positive electrode. In this way the mobility of nanocrystal–oligonucleotide conjugates is higher, the more oligonucleotides are bound per nanocrystal. This is different from what was described above for gold–oligonucleotide conjugates, since phosphine coated Au nanocrystals are negatively charged, but PEG silanized CdSe/ZnS nanocrystals are almost neutral [128]. If oligonucleotides modified with an organic dye, such as Cy5, are bound to the nanocrystals, further control of the conjugation is possible. If green fluorescent nanocrystals are conjugated with Cy5-modified oligonucleotides and the conjugates are run on an agarose gel, the position of the

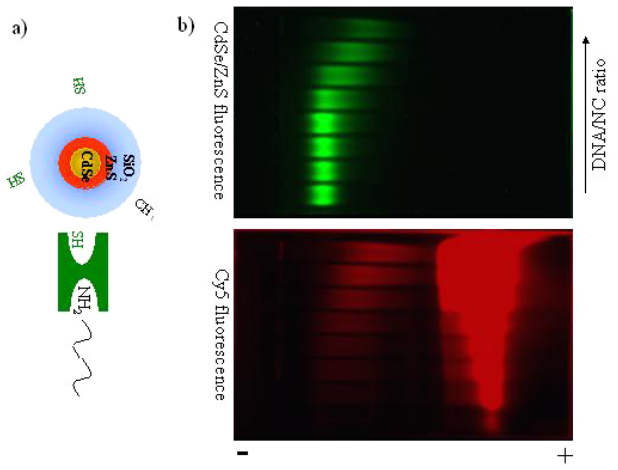

Figure 5. Conjugation of CdSe/ZnS nanocrystals with oligonucleotides. (a) Amine ($-NH_2$) modified oligonucleotides can be conjugated with silanized CdSe/ZnS nanocrystals bearing thiol (–SH) groups on their surface with bifunctional crosslinker molecules. (b) Separation of nanocrystal–oligonucleotide conjugates with gel electrophoresis. 30 base oligonucleotides are bound in different stoichiometric amounts to silanized CdSe/ZnS nanocrystals and are incubated for several hours. The oligonucleotides are modified with an organic fluorophore (Cy5) that emits red fluorescence. The green fluorescent nanocrystals are stabilized with polyethylene groups and are therefore almost neutral. The samples are loaded in the wells of a 1% agarose gel (close to the '−' electrode on the left side of the images). A voltage (100 V $\Leftrightarrow$ 6.7 V cm^{-1}) is applied along the gel for 1 h. The conjugates migrate towards the '+' electrode. The higher the oligonucleotide (DNA) to CdSe/ZnS nanocrystal ratio, the higher the mobility of the conjugates becomes. Two images of the gel are shown: the upper one shows the green fluorescence of the nanocrystals, the lower one the red fluorescence of the Cy5-modified oligonucleotides. The nanocrystal and oligonucleotide fluorescence is colocalized, which proves that the oligonucleotides are attached to the particles. Unmodified particles (lowest lane) migrate more slowly than particle–oligonucleotide conjugates. However, the binding process is not very efficient, as can be seen in the lower image: a lot of oligonucleotides do not bind to the particles; they can be seen as a rapidly migrating red fluorescent band on the right side of the image.

nanocrystals and the oligonucleotides in the gel can be detected separately: the nanocrystals emit green fluorescence light and the Cy5-modified oligonucleotides red fluorescence light. Free, unbound oligonucleotides migrate much faster than nanoparticle–oligonucleotide conjugates and they are located at positions on the gel with solely red fluorescence. Free nanocrystals barely migrate and they are located at positions on the gel with solely green fluorescence. Nanoparticle–oligonucleotide conjugates migrate faster than plain nanocrystals, but slower than free oligonucleotides. They can be identified on the gel by the colocalization of green and red fluorescence [128]. In this way the attachment of oligonucleotides to silanized semiconductor nanocrystals can be clearly proven. Unfortunately, silanized CdSe/ZnS nanocrystals are not very homogeneous in terms of their charge and size distribution. The bands of silanized CdSe/ZnS nanocrystals on agarose gels are therefore much broader than that of phosphine coated Au nanocrystals (see figures 4 and 5). Due to these broad bands, no discrete bands for CdSe/ZnS–oligonucleotide conjugates have been obtained so far, although this is possible for Au–

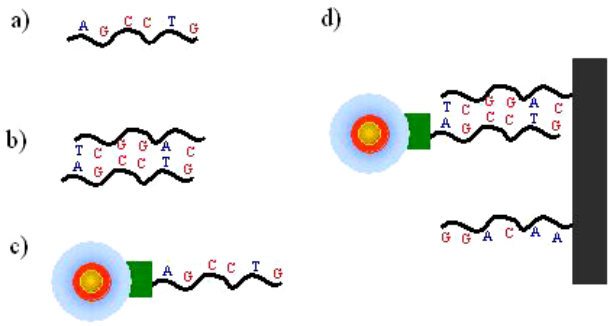

Figure 6. DNA as a molecular template to arrange nanoscale objects. (a) One oligonucleotide composed of six bases (A, G, C, C, T, G). (b) One oligonucleotide (AGCCTG) bound to a complementary oligonucleotide. (c) Conjugate formed between a silanized CdSe/ZnS nanocrystal and an oligonucleotide with six bases. (d) The nanocrystal–oligonucleotide conjugate binds to an oligonucleotide with complementary sequence that is immobilized on a surface, but does not bind to oligonucleotides with different sequences.

oligonucleotide conjugates. Therefore, it has not yet been possible to obtain colloidal CdSe/ZnS nanocrystals with a controlled number of oligonucleotides attached per particle.

4. Some applications

4.1. Molecular recognition

One of the most fascinating capabilities of many biological molecules is that of molecular recognition [18, 152–156]. Certain biological molecules can recognize and bind to other molecules with extremely high selectivity and specificity. This is similar to a lock-and-key system, but on a molecular level. Out of millions of keys, or ligand molecules, only one fits a particular lock, or receptor molecule. Concerning molecular recognition, two classes of biological molecules are of special interest: antibodies and oligonucleotides. Antibodies are protein molecules created by the immune systems of higher organisms [152]. If, for example, a virus enters an organism, antibodies will recognize the virus as a hostile intruder, or antigen, and bind to it in such a way that the virus can be destroyed by other parts of the immune system. It is very important that antibodies be able to distinguish between the tissue and molecules that are part of the host organism and antigens coming from outside; failure to do so results in autoimmune diseases that cause destruction of tissue from the host organism. Antibodies therefore have to be highly selective and they have to distinguish between millions of molecules. There is basically an antibody available for each possible antigen that binds only to this antigen.

Oligonucleotides are linear chains of nucleotides, also called single-stranded DNA (deoxyribonucleic acid). Each nucleotide is comprised of a sugar backbone and a base. There are four different bases: adenine (A), cytosine (C), guanine (G) and thymine (T) [152]. Each oligonucleotide is characterized by the sequence of its bases. One six-base oligonucleotide could, for example, have the sequence AGCCTG, another one the sequence AACAGG; basically, all permutations are possible (see figure 6). In order to indicate the direction, e.g. which base is the start and which is the

end of the chain, the sequence is written as 5′-AGCCTG-3′. The key point is that A can bind to T and C can bind to G, but A does not bind to C or G, etc. Therefore, the oligonucleotide sequence 5′-AGCCTG-3′ binds to the oligonucleotide sequence 5′-CAGGCT-3′ (see figure 6). Since there is a direction, the start (5′) of one oligonucleotide always binds to the end (3′) of the other oligonucleotide. In the case of 5′-AGCCTG-3′ and 5′-CAGGCT-3′ the A, G, C, C, T, G of the first oligonucleotide binds to the T, C, G, G, A, C of the second oligonucleotide, respectively (see figure 6). The binding of the bases is called Watson–Crick base pairing or hybridization [152]. 5′-AGCCTG-3′ and 5′-CAGGCT-3′ are complementary oligonucleotides and both oligonucleotides bound together are called double-stranded DNA. The more complementary two oligonucleotides are, the more tightly they bind to one another. For example, the binding of one oligonucleotide to another in which all but one base is complementary, a so-called single base mismatch or single nucleotide polymorphism (SNP), is significantly weaker than a fully complementary oligonucleotide. The binding of oligonucleotides, therefore, is highly selective and specific. Since the molecular recognition is based on the sequence of the bases, it is possible to design code sequences with enormous variety.

In addition to the antibody–antigen and oligonucleotide–complementary oligonucleotide systems, the avidin (streptavidin)–biotin system is often used as a receptor–ligand pair [157–159].

Through the process of binding biological molecules such as antibodies, single-stranded DNA and streptavidin to the surface of water-soluble nanocrystals, the principle of molecular recognition can be used for many experiments. When a nanocrystal is conjugated to a receptor molecule it is ‘tagged’. The nanocrystal–receptor conjugate now specifically binds with high sensitivity and selectivity to ligand molecules that ‘fit’ the molecular recognition of the receptor (see figures 6(d) and 9(a)). In other words, nanocrystals conjugated with a receptor can be ‘directed’ to bind to positions where ligand molecules are present. This facilitates a set of different applications [160, 161].

Although there are many more possible applications, such as using nanocrystal labelled microspheres as colour codes to identify many molecules in parallel [15], the next section of this review will focus on three particular applications.

(i) If one type of nanocrystal is conjugated with a receptor molecule, and another type is conjugated with the respective ligand molecule, groupings of both types can be formed. One example would be to conjugate red fluorescent nanocrystals to one oligonucleotide and green fluorescent nanocrystals to the complementary oligonucleotide, and then hybridize the DNA and thus form dimers of red and green fluorescent nanocrystals.

(ii) Another application is to arrange nanocrystals on a surface. If ligand molecules are immobilized on a surface, receptor-conjugated nanocrystals will bind to these positions. One futuristic idea is to use this idea for the realization of a nano-circuit. Single nanocrystals can be used as single electron transistors [162]. However, without a technique to arrange and connect these electronic building blocks no nanoscale circuit can be created. Structuring a surface with a programmable DNA pattern and hybridizing it with nanocrystal–DNA conjugates could arrange the nanocrystals in a desired pattern of the surface [163]. In a subsequent step, parts of the DNA molecules could be metallized [38] and thus electric wires connecting the transistor could be formed.

(iii) Finally, biomolecule conjugated nanocrystals can be used as labels. Semiconductor nanocrystal–oligonucleotide conjugates, for example, could be used as fluorescence labels for DNA chips [164–166]. DNA and streptavidin conjugated semiconductor nanocrystals can be used as fluorescent probes for targeting sequences in the nucleus [7] or structural elements [5] of cells, respectively.

4.2. DNA mediated groupings of nanocrystals

Nanoscale particle assemblies using the avidin (streptavidin)–biotin receptor–ligand or similar systems have been demonstrated for a variety of different nanoparticles, including polymer microspheres and gold nanocrystals [167–174]. Modifying nanocrystals with single-stranded DNA allows for even more flexibility to form different groupings between the particles. If gold nanocrystals of two different sizes are conjugated with complementary oligonucleotides, aggregates of gold nanocrystals are formed after hybridization of the DNA [134, 135, 141, 175, 176]. The base pairing can be reversed by heating, which can be seen as a colour change in the solution. If oligonucleotide-conjugated nanocrystals bind close to each other on a long single-stranded DNA molecule, the colour of their absorption changes from red to blue [139, 140]. Using the same concept, controlled multilayers of gold nanoparticles can be formed [141]. With care, it is possible to create a small, defined structure of gold nanocrystals, for example, dimers of one big and one small gold particle, or trimers of three particles [98] (see figure 7). However, the yield in obtaining defined structures is currently relatively low. One reason is that, previously, the structures were generated without precisely controlling the number of DNA molecules attached per gold particle [98]. For the formation of a gold particle trimer, the particle in the middle must be conjugated with exactly two oligonucleotides, otherwise incomplete or wrong structures are formed as side products. Using DNA–gold conjugates purified with gel electrophoresis and thus with a controlled number of oligonucleotides per particle [143] should result in significantly higher yield. Besides dimers and trimers [98, 177], no structures of higher complexity have been reported so far. In addition, most experiments of defined groupings have been done with gold particles. So far, no defined particle groupings of mixed particles, such as dimers between gold and semiconductor nanocrystals, have been reported, although aggregates of gold and semiconductor particles have been demonstrated [112].

4.3. DNA mediated arrangement of nanocrystals on a surface

A feature of many biological molecules is their ability to self-assemble. Self-assembly means that molecules mixed together will spontaneously assemble in a structured, regular pattern, in order to minimize the total free energy [26, 29, 30, 178]. Self-assembly is not limited to biological molecules and can

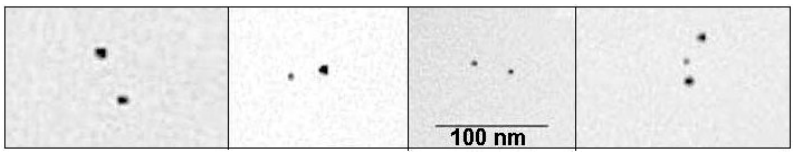

Figure 7. Groupings of Au nanocrystals connected by DNA. Gold nanocrystal–oligonucleotide conjugates with complementary sequences are mixed and incubated. The complementary sequences hybridize to double-stranded DNA and small groupings of particles are generated. Low resolution TEM images of groupings of (a) two 10 nm diameter, (b) one 5 and one 10 nm, (c) two 5 nm and (d) two 10 nm and one 5 nm Au nanocrystals are shown.

be triggered by several factors: for molecules or objects with regular geometry it often is desirable to arrange in a regular pattern [75, 91, 109, 179]. Small molecules such as hydrocarbon chains or lipids often spontaneously arrange to form complex regular structures, caused by hydrophobic/hydrophilic and electrostatic interactions; prominent examples are Langmuir–Blodgett (LB) films and self-assembled monolayers (SAM) on surfaces [180–185]. Furthermore, self-assembly can be initiated by molecular recognition. By combining at least four different types of single-stranded DNA molecules that are partly complementary to each other, two-dimensional DNA lattices form [35, 186].

If nanocrystals are deposited on a structured sample surface, the positions of nanocrystal adsorption are in many cases oriented, i.e. they 'follow' the pattern of the surface. In the simplest case the surface of the unmodified substrate is already well oriented, such as for highly oriented pyrolytic graphite (HOPG), and the nanocrystals' arrangement is dictated by this orientation [187]. A friction transfer of carbon films can also mechanically structure the surface of a substrate in the nanometre regime. Nanocrystals then self-assemble parallel to the carbon lines [188]. Nanocrystals also follow the orientation of self-assembled polymer films [189]. However, in order to obtain more complex arrangements of nanocrystals on a surface, ways have to be found to pattern surfaces in a more controlled way. This can be done by the self-assembly of biological molecules on the surface. Due to the high complexity of biological molecules and their ability for molecular recognition, complex patterns can be formed, such as from modified porphyrin molecules [30] or proteins [190, 191]. The approach with the most programmability certainly is the self-assembled formation of two-dimensional DNA crystals on a surface [35, 186]. Other techniques allow for the direct patterning of surfaces with biomolecules by mechanical manipulation [192, 193]. However, these approaches are sequential and not parallel like pattern creation in a self-assembly process.

The general concept now is the following: first, a surface is patterned with biological molecules, preferably using self-assembly. By varying the properties of the molecules (e.g. selecting the sequence of oligonucleotides) the pattern can be controlled. The surface is then incubated with biomolecule-modified nanocrystals. The biological molecules bound to the nanocrystals (receptor) now recognize their ligand in the pattern on the surface of the substrate. In this way the nanocrystals are directed with high specificity and selectivity to their desired positions on the substrate. As an example, regular patterns of oligonucleotides can be self-assembled on a surface in a programmable way, just by using different sequences. It is important that some free, unpaired bases remain (sticky ends). If oligonucleotide–nanocrystal conjugates are then added, the oligonucleotides attached to the nanocrystals bind to the unpaired bases of the oligonucleotide pattern adsorbed on the surface. The DNA acts as a template or scaffold for the arrangement of the nanocrystals [22, 36].

DNA directed arrangement of nanocrystals on a surface can be nicely demonstrated with a sorting experiment. Different parts of a surface are patterned with oligonucleotides of different sequences. Different types of nanocrystals are conjugated with oligonucleotides that are complementary to the oligonucleotides immobilized at particular positions on the substrate. The surface is incubated with the oligonucleotide–nanocrystal conjugates and rinsed after hybridization. The principle of DNA directed arrangement is proven when nanocrystals only bind to the part of the surface that is immobilized with oligonucleotides complementary to that conjugated to the nanocrystal (see figure 8). DNA mediated sorting has been demonstrated with Au [194] as well as with CdSe/ZnS nanocrystals [166]. In both cases, selectivity is high enough to clearly discriminate single nucleotides polymorphisms.

Also based on the principle of molecular recognition are immunoassays involving nanocrystals. A surface is patterned with antigens of different concentrations and incubated with antibody-conjugated nanocrystals. After rinsing, only the patterned areas of the surface are covered with nanocrystals, where the antibody–nanocrystal conjugate is bound to a matching antigen. The nanocrystal coverage can be measured either by absorption, as for Au nanocrystals [195], or by fluorescence, as for CdSe/ZnS nanocrystals [115]. The concentration of antigens can be determined by the content of nanocrystal coverage of the respective areas.

4.4. Biomolecule–nanocrystal conjugates as fluorescence label for biological cells

Fluorescence labelling of specific compartments in cells is a widely used method in biology to visualize structural units that, due to a lack of contrast or resolution, cannot be distinguished by just recoding an image. The idea is to chemically link a fluorescent dye to receptor molecules that specifically and selectively bind to the desired compartment in the cell (see figure 9). This receptor molecule typically is an antibody against the structure to be labelled. Another very popular receptor is avidin or streptavidin: first, the structure to be labelled is incubated with a biotinylated antibody, which is then recognized by the dye–avidin construct. Often, even more complex labelling schemes involving multiple steps and secondary antibodies are employed [152, 196]. In order to label gene sequences in the nucleus of a cell,

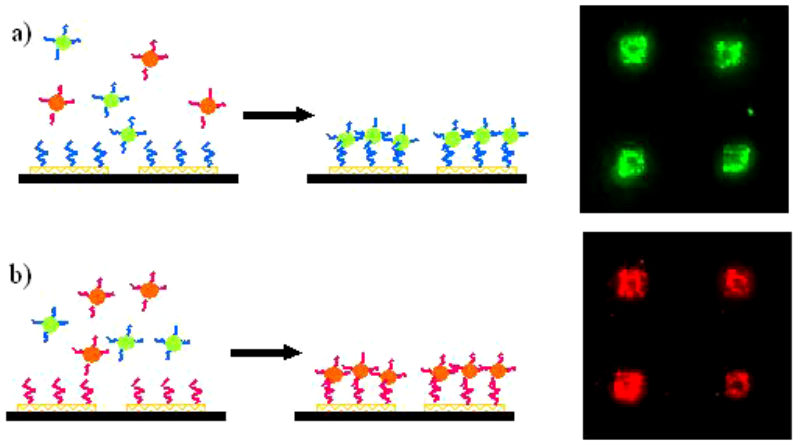

Figure 8. DNA mediated arrangement of CdSe/ZnS nanocrystals on a surface. Red and green fluorescent silanized CdSe/ZnS nanocrystals are conjugates with two different oligonucleotide sequences (21 bases). Gold squares on a glass substrate (8 μm length) are immobilized with complementary oligonucleotides. The gold surface in (a) and (b) is coated with oligonucleotides complementary to the green and red fluorescent nanocrystal–oligonucleotide conjugates, respectively. The gold surfaces are exposed to a solution containing both the green and the red fluorescent particle–oligonucleotide conjugates. After incubation several rinsing steps remove unbound conjugates. On the right side fluorescence images obtained with an optical microscope of the gold squares are shown. Only particles conjugated to oligonucleotides that have the complementary sequence to the oligonucleotides immobilized on the gold surface bind.

oligonucleotide–dye constructs are used (FISH: fluorescence *in situ* hybridization). In addition to organic fluorophores, fluorescent silica nanospheres [197, 198] are typically used as dyes. Due to their properties, fluorescent semiconductor nanocrystals offer several advantages compared to other dyes and therefore have already been used in labelling experiments (see figure 9) [5–8, 114, 151]. Since all colours of emission can be excited with a single wavelength (preferably UV) and the bandwidth of the emission spectra is narrow, many colours can be used in parallel. In the most pessimistic case, assuming a bandwidth of 40 nm, at least five different colours of emission can be individually distinguished (e.g. 500, 540, 580, 620 and 660 nm). All of these colours originate from the same type of fluorescent dye: the nanocrystals are identical but for their size. By labelling different sizes of nanocrystals with different antibodies, multicolour staining of several compartments in cells should be possible. So far, only two colours in parallel have been demonstrated, but there is the potential for at least five. Furthermore, compared to many organic dyes, nanocrystals suffer less from photobleaching [88], which suggests their use for labelling of dynamic processes. In addition, the decay time of the fluorescence of nanocrystals is longer than that of typical organic dyes and, most importantly, also longer than the decay time of autofluorescence. In this way, using time-gate imaging can reduce the autofluorescence background in fluorescence imaging of cells [199, 200].

4.5. Phagokinetic tracks

In contrast to the applications described so far that involve nanocrystals conjugated with biological molecules, the application described in this paragraph is based on plain, unconjugated water-soluble nanocrystals. When living cells are exposed to fluorescent silanized CdSe/ZnS nanocrystals they incorporate or eat the nanocrystals [201]. The detailed mechanism of this process remains unclear, but the pathway to take up nanocrystals most likely is endo-, pino- or phagocytosis. Inside the cell the incorporated nanocrystals are

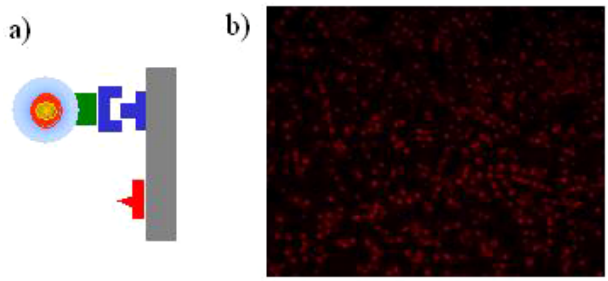

Figure 9. Fluorescence labelling of cells. (a) A biological molecule is attached to the surface of a silanized CdSe/ZnS nanocrystal. This receptor molecule binds specifically to certain ligand molecules. If a surface bearing several different ligand molecules is exposed to nanocrystals conjugated with receptor molecules, the nanocrystals will be directed to an area of the surface where the receptor and ligand molecules bind in a manner similar to the lock-and-key principle. (b) Staining of nuclear antigens on mouse kidney sections using red fluorescent streptavidin coated CdSe/ZnS nanocrystals that bind to the biotinylated antibodies. The size of the image recorded with an optical microscope is 210 μm $\times$ 170 μm (courtesy of Dr M Bruchez, Quantum Dot Corp., 26118 Research Road, Hayward, CA, USA, http://www.qdots.com).

collected and stored in vesicular compartments (see figure 10). The uptake of nanocrystals seems to have minimal impact on the cells; they keep on migrating, dividing, etc [201]. Even water-soluble gold nanoparticles a few hundred nanometres in diameter are incorporated by living cells, although the pathway of ingestion for these big particles might be different from those of the small silanized CdSe/ZnS particles with diameters of 5–15 nm.

A quarter of a century ago Albrecht-Buehler developed a mobility assay for living cells based on the uptake of nanoparticles [202–204]. The principle is very simple: the surface of a glass substrate is homogeneously coated with colloidal gold nanoparticles. Cells are then seeded on this layer of nanocrystals. As already described, the cells ingest the nanoparticles. While the cells migrate on the substrate,

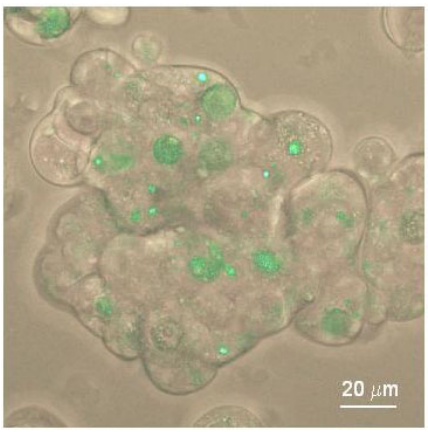

Figure 10. Uptake of silanized CdSe/ZnS nanocrystals by living cells. Nanocrystals (green fluorescence) have been incorporated by MCF-10A cells that form a cluster.

they eat all underlying particles, leaving behind a particle-free trail. In other words, the area cleared of nanocrystals is a blueprint of the pathway on which the cells have moved on the substrate. The gold particles originally used in these assays were quite inhomogeneous and large (a few hundred nanometres), considering that the typical size of a cell is of the order of a few tens of microns. Therefore, using silanized CdSe/ZnS nanocrystals instead of colloidal gold for this assay, called phagokinetic tracks, offers several advantages. Besides being an order of magnitude smaller and more homogeneous, CdSe/ZnS nanocrystals can be visualized by their fluorescence, in contrast to the absorption that is necessary for Au colloids. If any cell culture substrate is coated with a layer of red fluorescent nanocrystals, for example, the surface of the substrate fluoresces homogeneously in red. Once cells are seeded on the substrate, they start to migrate while eating nanocrystals. Therefore, there is no fluorescence anywhere cells have passed, and the pathway of the cells is visible as black trails within a red fluorescent background (see figure 11). Besides being an assay for investigating the migration of cells, this assay might be used to distinguish between cancerous and non-cancerous cells. In general, cancer cells eat nanocrystals faster and migrate more rapidly on a substrate. The higher the metastatic potential of a cell is, the faster they clear larger areas of nanocrystals. The area of the substrate cleared from nanocrystals by one cell, as measured by the size of the non-fluorescent area, therefore can provide a measure of the metastatic potential [201]. However, so far only the principle of the assay has been demonstrated; therefore, before this is applicable, many statistical investigations remain to be done.

5. Summary

The synthesis of nanocrystalline objects is possible for multiple materials with controlled size and controlled shape. Although synthesis is possible in polar solvents, synthesis in non-polar solvents generally results in better particles. The synthesized hydrophobic nanocrystals first have to be converted to particles with a hydrophilic surface in order to be water-soluble. Water-soluble nanocrystals can be conjugated with many biological molecules. Based on the principle of molecular recognition

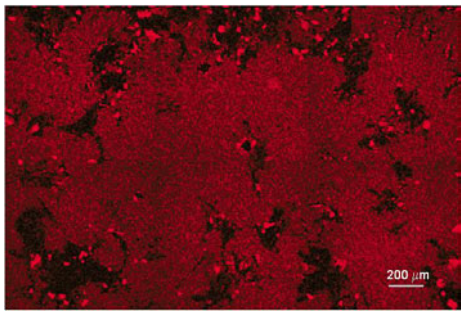

Figure 11. Phagokinetic tracks. MDA-MB-231 cells were seeded on a layer of red fluorescent nanocrystals. The cells migrate along the surface, incorporate nanocrystals, leaving behind a black trail along their pathway.

nanocrystal–biomolecule conjugates bind to specific sites and thus can be directed. In this way it is possible to arrange nanocrystals in a programmable way and to use fluorescent nanocrystals as dyes for biological labelling. Since living cells ingest nanocrystals, the uptake of the particles from a surface can be used for a migration assay (phagokinetic tracks). Although some applications have been studied, as demonstrated in this review, the use of colloidal nanocrystals in life sciences is still in its beginnings [205].

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